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Ferroportin-mediated mobilization of ferritin iron precedes ferritin degradation by the proteasome

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Ferritin is a cytosolic molecule comprised of subunits that self-assemble into a nanocage capable of containing up to 4500 iron atoms. Iron stored within ferritin can be mobilized for use within cells or exported from cells. Expression of ferroportin (Fpn) results in export of cytosolic iron and ferritin degradation. Fpn-mediated iron loss from ferritin occurs in the cytosol and precedes ferritin degradation by the proteasome. Depletion of ferritin iron induces the monoubiquitination of ferritin subunits. Ubiquitination is not required for iron release but is required for disassembly of ferritin nanocages, which is followed by degradation of ferritin by the proteasome. Specific mammalian machinery is not required to extract iron from ferritin. Iron can be removed from ferritin when ferritin is expressed in *Saccharomyces cerevisiae,* which does not have endogenous ferritin. Expressed ferritin is monoubiquitinated and degraded by the proteasome. Exposure of ubiquitination defective mammalian cells to the iron chelator desferrioxamine leads to degradation of ferritin in the lysosome, which can be prevented by inhibitors of autophagy. Thus, ferritin degradation can occur through two different mechanisms.

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Introduction

Sequestration of iron within ferritin is the mechanism by which most eukaryotic cells store iron. Cells can mobilize iron from ferritin for export and for heme synthesis (Peto *et al*, 1983). The mechanisms by which iron regulates ferritin synthesis and by which iron enters ferritin have been well characterized (Liu and Theil, 2005). In contrast, the mechanism(s) by which iron exits ferritin is unclear. Studies have shown that reductants in the presence of iron chelators can lead to iron loss from purified ferritin (Harrison *et al*, 1974) and that engineered amino-acid substitutions in recombinant ferritin molecules can accelerate iron loss (Takagi *et al*, 1998). These studies suggesting that iron exits ferritin through a pore in the assembled ferritin nanocage. These studies, however, have relied solely on *in vitro* analyses and there is no evidence that iron can exit ferritin prior to ferritin degradation. Other studies have shown that cytosolic ferritin gains entry into lysosomes and that ferritin degradation within lysosomes is responsible for iron release. The lysosomal degradation of ferritin has been seen in cells induced for autophagy by amino-acid starvation (Harrison *et al*, 1974), in cells infected with *Neisseria* (Larson *et al*, 2004), in cells exposed to drugs such as the iron chelators (Bridges, 1987; Konijn *et al*, 1999; Kidane *et al*, 2006) and the anticancer agent doxorubicin (Kwok and Richardson, 2004) or in cells given a high iron load through the administration of cationic ferritin (Radisky and Kaplan, 1998). Iron release from ferritin could be blocked by inhibiting lysosomal proteolysis, either by increasing lysosomal pH or through addition of protease inhibitors. Entry of ferritin into lysosomes may, however, reflect a response to cellular stresses, rather than a physiological route for iron release.

To evaluate the role of lysosomes in ferritin iron release, we expressed the plasma membrane iron exporter ferroportin (Fpn). Fpn is a cell surface iron transporter present on all cell types that export iron into plasma. Mutations in Fpn lead to cellular iron accumulation, most of which is in ferritin (Ganz and Nemeth, 2006). We have utilized the regulated expression of Fpn to examine the mechanism by which iron exits ferritin. Our results show that degradation of ferritin is not required for mobilization of iron and that iron-poor ferritin is degraded in the cytosol through the action of the proteasome. Monoubiquitination of ferritin nanocages is required for ferritin disassembly. Monoubiquitination and proteasomal ferritin degradation can also be seen when ferritin is expressed in *Saccharomyces cerevisiae,* a species that does not contain endogenous ferritin. These results show that iron exit from ferritin, like iron entry into ferritin, is an autonomous property of the ferritin nanocage.

Results

Fpn-mediated ferritin degradation occurs in the cytosol

Expression of Fpn leads to decreased cytosolic iron and reduced levels of ferritin (Nemeth *et al*, 2004). To determine if expression of Fpn leads to the lysosomal degradation of ferritin, cells stably transformed with an ecdysone regulated Fpn-GFP were incubated with ferric ammonium citrate (FAC) as a source of iron and, in the continued presence of FAC, with the ecdysone analog Ponasterone A to induce expression of Fpn-GFP. Cells exposed to FAC accumulate large amounts of ferritin as determined by enzyme-linked immunosorbent assay (ELISA), while expression of Fpn-GFP led to the loss of

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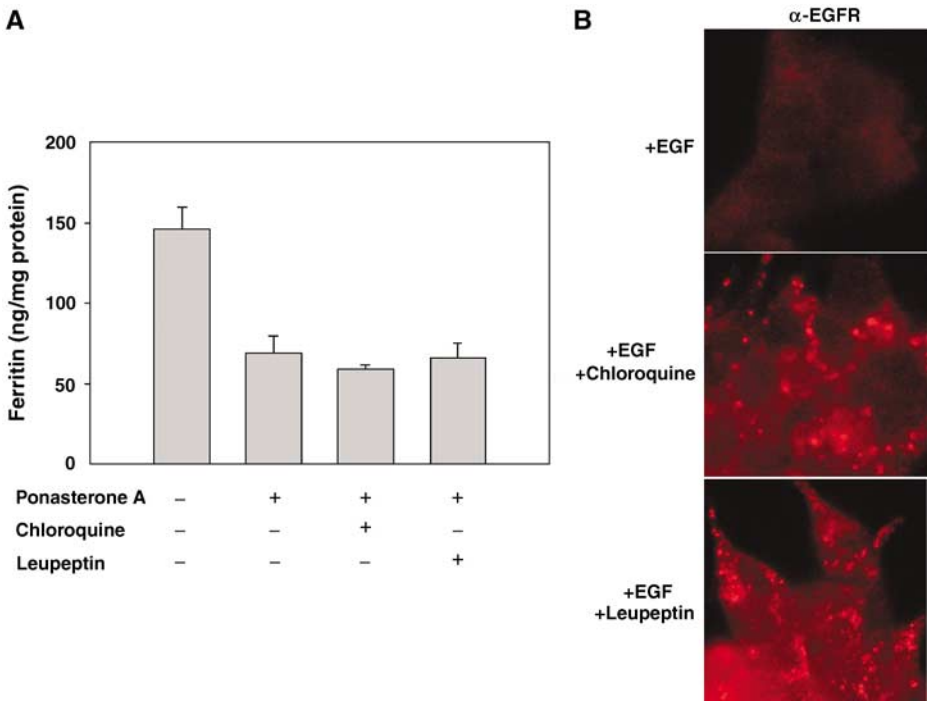

Figure 1 Fpn-mediated decrease in ferritin is not prevented by chloroquine or leupeptin. (**A**) HEK293T-Fpn cells were incubated with FAC (10 μM Fe) for 24 h followed by incubation for 6 h in the absence or presence of 10 μM Ponasterone A. Cells were then incubated with and without 100 μM chloroquine or 10 μM leupeptin for 10 h and harvested. The ferritin content was determined by ELISA. Induction of Fpn resulted in decreased ferritin levels and this was not prevented by treatment with chloroquine or leupeptin. The data are presented as the standard deviation from three different experiments. (**B**) Cells treated as in (A) were incubated in the presence of 1 μg/ml EGF for 2 h. Cells were fixed and processed for immunofluorescence using mouse anti-EGF receptor and Alexa 594 conjugated goat anti-mouse IgG. Chloroquine and leupeptin effectively inhibit degradation of EGF receptor.

accumulated ferritin (Figure 1A, columns 1 and 2). The Fpn-GFP-mediated decrease in ferritin was not prevented by chloroquine (Figure 1A, column 3) or leupeptin (Figure 1A, column 4). These agents prevent lysosomal proteolysis either by increasing lysosomal pH (chloroquine) or inhibiting lysosomal proteases (leupeptin). To confirm that these agents inhibited lysosomal proteolysis, we added epidermal growth factor (EGF) to cells, which results in the internalization of the EGF–EGF receptor complex and subsequent degradation of the complex in lysosomes. Addition of EGF to control cells resulted in loss of the EGF receptor (Figure 1B). Addition of EGF to either chloroquine or leupeptin-treated cells led to the disappearance of the receptor from the cell surface and its accumulation in late endosomes/lysosomes (Figure 1B). Since chloroquine and leupeptin do inhibit lysosomal proteolysis, we attribute the loss of ferritin to non-lysosomal proteolysis.

These results suggest that upon induction of Fpn-GFP, ferritin remains in the cytosol even as cells are being depleted of iron. We confirmed this result by examining the subcellular distribution of ferritin. Fpn was induced in iron-loaded cells. The cells were homogenized, the homogenate fractionated on a Percoll gradient and the distribution of lysosomes and ferritin determined (Supplementary Figure 1). The lysosomal enzyme β-*N*-acetylhexoseaminidase showed two peaks of activity, neither of which was coincident with ferritin. We note the presence of a single point of ferritin in fraction three of the gradient. While the amount of ferritin decreased upon expression of Fpn-GFP, the subcellular distribution of ferritin was not affected even in cells incubated with leupeptin or chloroquine. These results support the conclusion that ferritin is not being degraded in lysosomes.

Cytosolic degradation of ferritin is proteasome-mediated

If ferritin is degraded in the cytosol, degradation by the proteasome would be likely. Addition of proteasome inhbitors MG132 or lactacystin to cells prevented the Fpn-GFP induced loss of ferritin (Figure 2A). For many proteins, a prerequisite for proteasome degradation is the covalent attachment of ubiquitin. To determine if ferritin is ubiquitinated, ferritin was immunoprecipitated and the immunoprecipitate examined for ubiquitin by Western blotting. There was no evidence of ubiquitinated ferritin in control cells, in cells incubated with FAC or in cells incubated with FAC and induced to express Fpn-GFP (Figure 2B). Ferritin was ubiquitinated in cells that had been incubated with FAC, induced to express Fpn and incubated with MG132. Immunopreciptiated ferritin contains both H and L chains as detected by Western analysis using H and L specific antibodies (data not shown). We are unable to determine whether both H and L chains are ubiquitinated, as these H and L specific antibodies do not work for immunoprecipitation. No evidence of an ubiquitin 'ladder' was seen and the increase in molecular mass of ferritin is consistent with monoubiquitination. We also noted the presence of a 40-kDa band that is detected with the anti-ferritin antibody (data not shown). This 40 kDa band was shown to be a crosslinked dimer of ferritin subunits (Mertz and Theil, 1983). The 40-kDa band was also ubiquitinated with what appears to be a single ubiquitin.

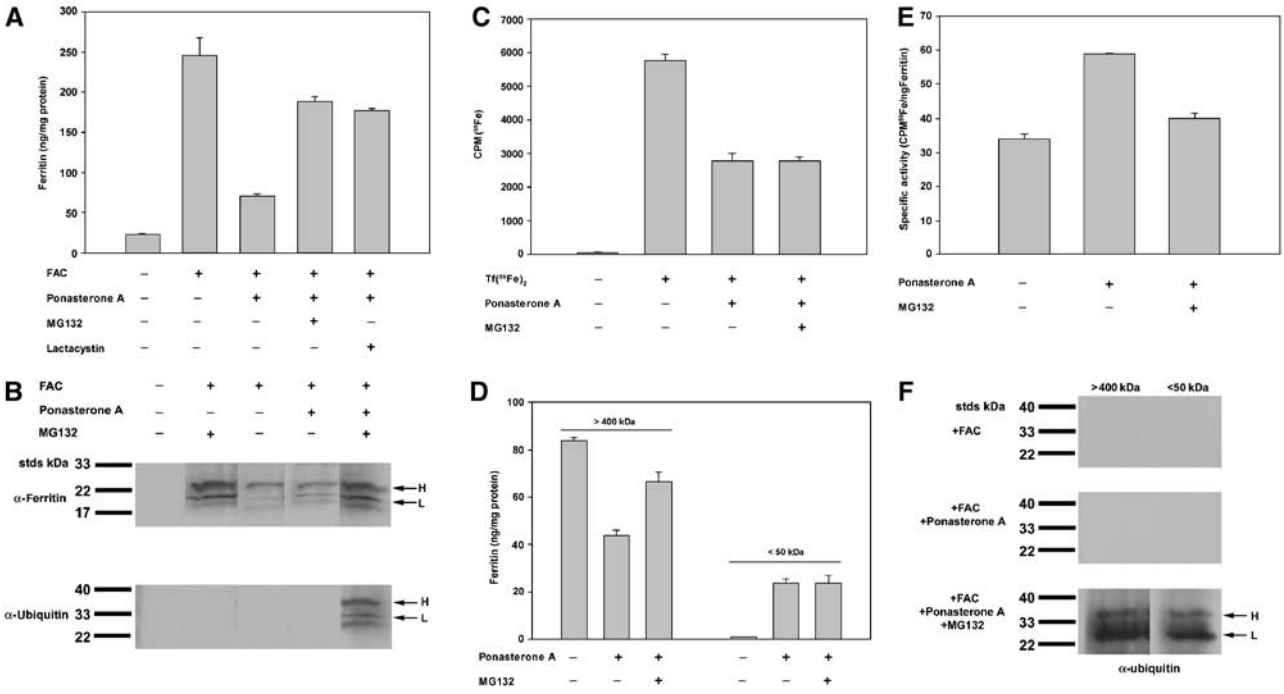

Figure 2 Fpn-mediated decrease in ferritin results from degradation by the proteasome. (**A**) HEK293T-Fpn cells were incubated with FAC (10 μM Fe) for 24 h followed by incubation for 6 h in the absence or presence of 10 μM Ponasterone A. Cells were then treated with or without 10 μM MG132 or 10 μM lactacystin in the presence of Ponasterone A for 10 h and harvested. The ferritin content was determined by ELISA. Error bars represent the standard deviation from three different experiments in duplicate. (**B**) Samples treated as in (A) were immunoprecipitated using anti-ferritin antibodies and the immunoprecipitate examined for the presence of ferritin or ubiquitin by Western blot analysis. The arrows indicate the migration of H and L chains. (**C**) Cells were incubated with 1.0×10^{-7} M Tf(^{59}Fe)$_2$ for 24 h followed by incubation for 6 h in the absence or presence of 10 μM Ponasterone A. Cells were then treated with or without 10 μM MG132 in the presence of Ponasterone A for 10 h and harvested and the amount of ^{59}Fe in immunoprecipitated ferritin was determined. (**D**) Cells were treated as described in (A) and cell extracts were applied to size exclusion chromatography and the ferritin content, in selected fractions, was determined by ELISA. Error bars represent the standard deviation from three different experiments in duplicate. (**E**) Cells were treated as described in (C), ferritin was then immunoprecipitated, eluted using 100 mM glycine, pH 2.5 and measured by ELISA. The amount of ferritin-associated ^{59}Fe was measured and the specific activity of ferritin determined. Error bars represent the standard deviation from three different experiments in duplicate. (**F**) Samples were treated as in (D), and ferritin was immunoprecipitated and analyzed by Western blot analysis using an antibody to ubiquitin.

Iron might be released after ferritin degradation or iron might exit ferritin followed by the degradation of the now iron-poor ferritin nanocage. To distinguish between these possibilities, cells were incubated with Tf (^{59}Fe)$_2$ to permit the accumulation of ^{59}Fe ferritin, and then incubated with Ponasterone A to induce Fpn-GFP, in the presence or absence of the proteasome inhibitor MG132. As shown above, induction of Fpn-GFP resulted in a loss of ferritin that was prevented by MG132. Immunoprecipitation of ferritin and measurement of ^{59}Fe showed that MG132 did not prevent the loss of ^{59}Fe from ferritin (Figure 2C). To exclude the possibility that MG132 leads to the accumulation of ferritin monomers, cell extracts were analyzed by size exclusion chromatography. Fractions were assayed for ferritin by ELISA, immunoprecipitated using antiferritin antibodies, and the amount of ^{59}Fe in the immunoprecipitate determined. In the absence of Fpn expression, only high molecular weight ferritin (>400 kDa) eluted from the size exclusion column (Figure 2D). In the absence of MG132, expression of Fpn-GFP led to a decrease in total cellular ferritin with an increase in low molecular weight ferritin, indicating that iron loss led to the disassembly of ferritin nanocages. Addition of MG132, which prevents ferritin loss, also led to the generation of disassembled ferritin. The specific activity of ^{59}Fe in the high molecular weight ferritin fraction was increased after induction of Fpn-GFP even though there was a decrease in total cellular ferritin (Figure 2E). Addition of MG132 to Fpn-GFP expressing cells inhibits ferritin degradation but not iron loss from ferritin, leading to the accumulation of iron poor ferritin. This result suggests that in the absence of the proteasome inhibitor, iron-poor ferritin is preferentially degraded.

We then determined if iron-poor ferritin nanocages disassemble first, followed by ubiquitination of free ferritin subunits or if ferritin nanocages are ubiquitinated. Ferritin was extracted from cells incubated with Ponasterone A and MG132 and ferritin nanocages were separated from ferritin subunits by size exclusion chromatography. The ubiquitination status of both ferritin nanocages and subunits were determined by Western blotting. Both assembled ferritin and ferritin monomers were ubiquitinated (Figure 2F), suggesting that ubiquitination occurs before subunit disassembly. Again, no evidence for polyubiquitination was seen on assembled or disassembled ferritin.

Release of iron from ferritin does not require ubiquitination

To determine if ubiquitination is required for iron release from ferritin, we utilized a cell line that has a temperature sensitive E1-ligase (Finley *et al*, 1984). Incubation of mutant cells (ts85) at the restrictive temperature inactivates the enzyme, decreasing the ability of cells to ubiquitinate target

proteins. Ts85 and parental cells (FM3A), however, do not express Fpn and once iron-loaded, both cell lines retain ferritin in the absence of iron (data not shown). To promote iron loss, cells were transformed with a plasmid expressing Fpn-GFP under the control of the CMV promoter. Expression of Fpn-GFP resulted in an inability of cells to accumulate iron or ferritin. Consequently, transformed cells were incubated with hepcidin to prevent iron export, thus permitting ferritin accumulation. Transformed cells were incubated with FAC for 24 h and then hepcidin was removed. Cells were then incubated at both permissive and restrictive temperatures. Fpn-GFP transformed FM3A or ts85 cells incubated with hepcidin accumulated similar amounts of ferritin and ferritin levels decreased when hepcidin was removed (Figure 3A). The decrease in ferritin was similar in FM3A cells incubated at the permissive and restrictive temperature and in ts85 cells at the permissive temperature. There was, however, an increased retention of ferritin in mutant cells at the restrictive temperature. Western analysis confirmed that ts85 cells incubated at the restrictive temperature were unable to ubiquitinate ferritin. While ts85 cells synthesize both H and L chains, we only detect one ubiquitinated ferritin band. We cannot determine whether this band is the H or L chain because the H and L specific antibodies cannot be used for immunoprecipitation (Supplementary Figure S2).

These results indicate that ubiquitination is required for ferritin degradation. The ts85 cells also permitted us to determine if ubiquitin addition is required for iron-release. Cells were treated as described above, but Tf(^{59}Fe)$_2$ was used to iron-load cells. Once iron loaded, hepcidin was removed to permit Fpn-mediated iron export and cells were incubated at the restrictive or permissive temperature. Ferritin levels were assayed by ELISA and ferritin immunoprecipitated to determine the amount of ^{59}Fe bound to ferritin. Removal of hepcidin resulted in a decrease in ferritin but an increase in ^{59}Fe-ferritin specific activity in ts85 cells incubated at the

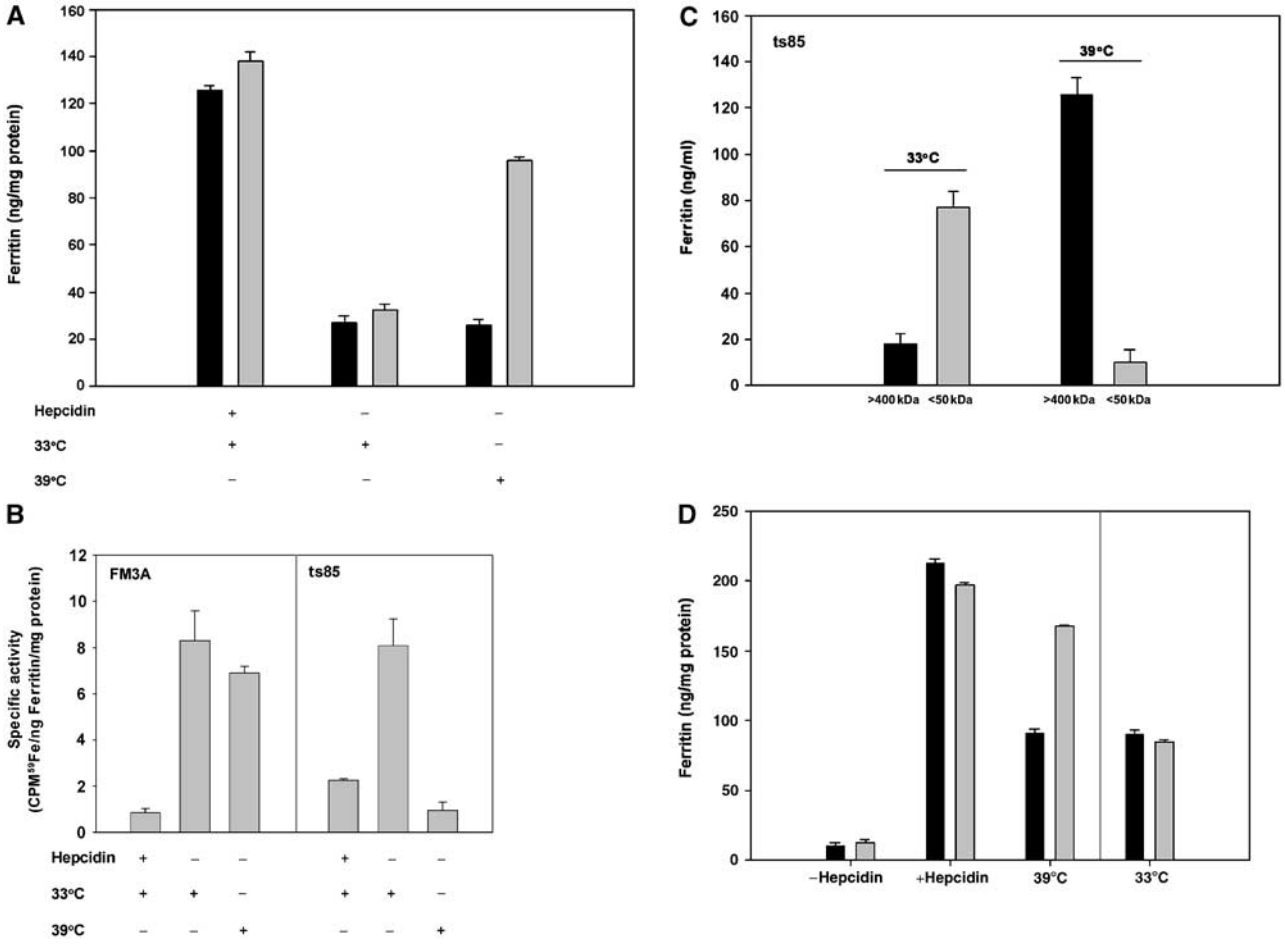

Figure 3 Ubiquitination is required for Fpn-mediated ferritin degradation. (**A**) FM3A (black) and ts85 (grey) cells were transiently transfected with a plasmid containing CMV-regulated Fpn-GFP and incubated in the presence of FAC (10 μM Fe) and 0.5 μM hepcidin for 24 h. Hepcidin was either maintained (+) or removed (−) to allow Fpn-GFP localization at the plasma membrane. Cells were maintained at the permissive temperature (33°C) or moved to the restrictive temperature (39°C) and incubated for 6 h. Cells were harvested and ferritin content determined by ELISA. Error bars represent the standard deviation from three different experiments in duplicate. (**B**) FM3A and ts85 cells were treated as in (A) but FAC was replaced with 1.0×10^{-7} M Tf(^{59}Fe)$_2$. Ferritin was immunoprecipitated and the specific activity of ^{59}Fe-ferritin determined. Error bars represent the standard deviation from three different experiments in duplicate. (**C**) ts85 cells were treated as in (B) and cell extracts applied to size exclusion chromatography. Ferritin content in selected fractions was measured by ELISA. Black bars represent assembled ferritin (>400 kDa), gray bars represent monomeric (<50 kDa). (**D**) FM3A and ts85 cells were treated as in (A) but cells that had been incubated at the restrictive temperature (39°C) were then returned to the permissive temperature (33°C) and incubated in the presence of cycloheximide (75 μg/ml) and 10 μM FAC for 1 h. Cells were then harvested and ferritin levels determined by ELISA. Error bars represent the standard deviation from three different experiments in duplicate.

permissive temperature and in FM3A cells incubated at either temperature (Figure 3B). This result again suggests that iron-poor ferritin is preferentially degraded before iron-rich ferritin. At the restrictive temperature, there was little loss of ferritin upon hepcidin removal in ts85 cells, consistent with the need to ubiquitinate ferritin for its degradation. There was, however, a decrease in ^{59}Fe-ferritin specific activity, indicating that although ferritin was not degraded, it became iron poor. These results show that ubiquitination is required for ferritin degradation but not for ferritin iron release. Size exclusion chromatography of ferritin, extracted from Fpn-GFP expressing ts85 cells incubated at the restrictive temperature, revealed that all of the ferritin was present as assembled cages, as there was little low molecular weight ferritin (Figure 3C). This result shows that ubiquitin addition, presumably to ferritin subunits, is required to disassemble ferritin nanocages.

To determine if iron could re-enter ferritin once it has been released, ts85 cells that had accumulated iron-poor ferritin at the restrictive temperature were then incubated at the permissive temperature. This was done under conditions in which iron export was prevented through addition of hepcidin, new ferritin synthesis was inhibited by addition of cycloheximide and iron loading promoted by the addition of FAC. In wild-type cells at either the permissive or restrictive temperatures, these additions prevented Fpn-mediated ferritin loss (Figure 3D, black bars). In ts85 cells that had been shifted from the restrictive to the permissive temperature, these additions did not prevent ferritin loss (Figure 3D, gray bars). If iron could reenter ferritin, then we might expect that ferritin would be stable at the permissive temperature. These results indicate that once iron has exited ferritin, the ferritin nanocage is marked for degradation and cannot readily re-accumulate iron.

Evidence for multiple routes of ferritin degradation

Our data show that Fpn-mediated ferritin iron release and degradation occurs in the cytosol. Studies by others have indicated that ferritin can be degraded in the lysosome (Bridges and Hoffman, 1986; Kidane *et al*, 2006). Most of those studies utilized the iron chelator DFO to induce ferritin iron loss. DFO is a high affinity Fe(III) chelator produced by *Streptomyces pilosus* that is used clinically to remove systemic iron in secondary iron overload disorders. We again took advantage of ts85 cells to determine if the mechanism of DFO-mediated iron loss was different than that of Fpn-mediated iron loss. Addition of DFO to iron-loaded ts85 cells led to the loss of ferritin even at the restrictive temperature (Figure 4A). Further, while MG132 did not prevent DFO-mediated ferritin loss, addition of chloroquine did prevent DFO-mediated ferritin loss.

It is likely that entry of ferritin into lysosomes might require an autophagocytic event. Sakaida *et al* (1990) showed that autophagy of ferritin led to the generation of a pool of iron that enhanced the cytotoxicity of hydroperoxides. Inhibition of autophagy by agents such as 3-methyladenine prevented that toxicity. Addition of 3-methyladenine to DFO-treated ts85 cells inhibited ferritin loss at the restrictive temperature (Figure 4B). This result suggests that DFO does induce autophagy of ferritin. Incubation of DFO-treated control cells with 3-methyladenine did not, however, prevent ferritin loss. This suggests that in the absence of autophagy,

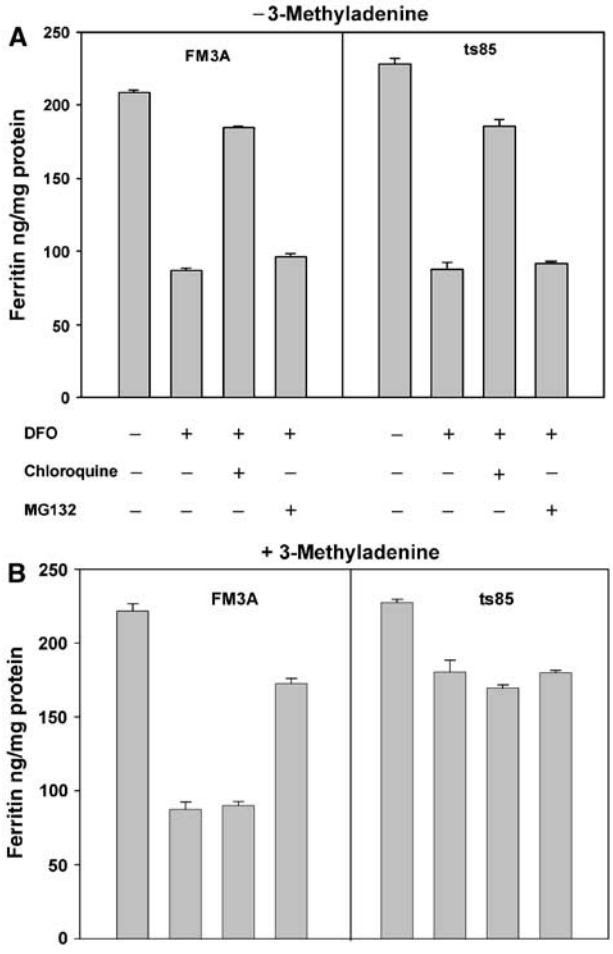

Figure 4 DFO leads to lysosomal degradation of ferritin. FM3A and ts85 cells were incubated in the presence of FAC (10 μM Fe) for 12 h. (**A**) Cells were then incubated at the restrictive temperature (39°C) for 6 h in the presence or absence of 100 μM DFO, with or without 100 μM chloroquine or 10 μM MG132. Cells were then harvested and ferritin content determined by ELISA. Error bars represent the standard deviation from three different experiments in duplicate. (**B**) Cells treated as in (A) were incubated in the presence or absence of 5 mM 3-methyladenine.

there may be an alternate route for ferritin degradation and that ts85 cells, which are defective in ubiquitination cannot access that pathway. This hypothesis was confirmed by examining the effect of the proteasome inhibitor MG132 on DFO-induced autophagy in control cells treated with DFO and 3-methyladenine. Addition of MG132 to 3-methyladenine treated control cells prevented ferritin degradation. These results indicate that ferritin degradation occurs by two routes: a DFO-induced entry of ferritin into lysosomes and a cytosolic route in which iron is extracted from ferritin prior to degradation by the proteasome.

Degradation of ferritin expressed in yeast occurs through the proteasome

To determine if specialized cellular machinery is required to extract ferritin iron in the cytosol, we expressed ferritin chains in *Saccharomyces cerevisiae*. Fungi, like plants, do not contain cytosolic ferritin, although plants have ferritin within organelles. Rather, fungi and plants store iron in vacuoles. Vacuolar membrane transporters mediate transfer

of iron from cytosol to vacuole and from vacuole to cytosol. Since yeast do not express ferritin, it is unlikely that they have a specialized mechanism to extract iron from ferritin. Kim *et al* showed that tadpole or human ferritin chains expressed in yeast could form iron-binding molecules (Shin *et al*, 2001; Kim *et al*, 2003). We confirmed and extended their results using genetic approaches to show that ferritin can store iron in yeast cytosol. First, expression of H and L chains or H chains under the control of the galactose inducible promoter (*Gal10*) led to increased cellular iron as measured by atomic absorption spectroscopy (Supplementary Figure 3A). Second, deletion of *CCC1*, which encodes the vacuolar iron importer, results in sensitivity of yeast to high iron (Li *et al*, 2001). Expression of ferritin under the galactose promoter protects Δ*ccc1* cells from high iron toxicity (Supplementary Figure 3B). Expression of ferritin lowered yeast cytosolic iron as shown by the induction of reporter constructs for a component of the high-affinity iron transport system (*FET3-lacZ*), which is transcriptionally activated by reduced levels of 'free' cytosolic iron (Supplementary Figure 3C). Induction of ferritin resulted in the presence of iron-loaded ferritin as defined by Prussian blue staining using nondenaturing gels (Supplementary Figure 3D). Thus, expression of H + L ferritin chains in yeast results in the synthesis of ferritin capable of binding iron.

If yeast that have accumulated ferritin are placed under conditions in which ferritin synthesis is prevented by the addition of glucose, ferritin levels remain high as long as cells are incubated in iron-rich medium (Figure 5A, closed circles). In the absence of continued ferritin expression, induction of the vacuolar iron transporter Ccc1p led to a reduction in ferritin levels (Figure 5A, closed triangles). These results show that ferritin levels decrease under conditions that promote low cytosolic iron. Decreased ferritin levels occur in cells that have compromised vacuolar proteolysis resulting from a deletion in the vacuolar protease *PEP4*, indicating that ferritin is not degraded in the vacuole (data not shown). Addition of MG132, however, prevented ferritin loss, suggesting that ferritin is degraded by the proteasome (Figure 5B). Immunoprecipitation of ferritin from MG132 treated cells showed the presence of ubiquitin on ferritin chains (Figure 5C). Again, there is no evidence of polyubiquitination. These results indicate that loss of iron from ferritin leads to ferritin ubiquitination and makes it unlikely that there is a specific iron-sensitive E3 ubiquitin ligase.

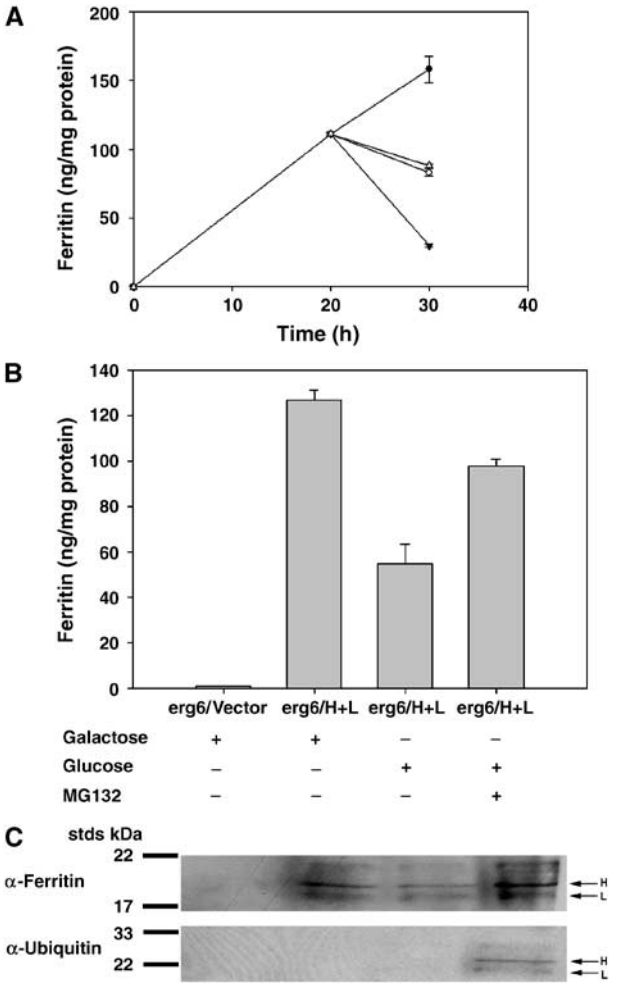

Figure 5 Human ferritin expressed in *S. cerevisiae* is degraded by the proteasome. Strains of wild type (Wt), Δ*ccc1*, *erg6-2* cells were transformed with plasmids pGAL, pGAL-L-ferritin, pGAL-H-ferritin and pGAL-H + L-ferritin. (**A**) Δ*ccc1*/pGAL-H + L cells were transformed with a plasmid containing a methionine regulated *CCC1* (pMET3CCC*1*). Cells were grown in medium with galactose for 20 h, washed and incubated in galactose (●), glucose with 10 × methionine (△), glucose (○) or glucose without methionine (▼) for 10 h. Cells were then harvested and ferritin levels determined by ELISA. Error bars represent the standard deviation from three different experiments in duplicate. The absence of methionine leads to expression of the vacuolar iron transporter Ccc1p. (**B**) *erg6-2* and *erg6-2* pGAL-H + L strains were grown in medium with galactose and 250 μM $FeSO_4$ for 24 h. Cells were then washed and incubated in galactose or glucose in the absence or presence of 50 μM MG132 for 7 h. (**C**) Cells were then harvested, ferritin levels determined by ELISA and immunoprecipitated using anti-ferritin antibodies and the immunoprecipitate examined for the presence of ubiquitin by Western analysis. Cells with the *erg6-2* allele permit the entry of MG132 (Lee and Goldberg, 1996).

Discussion

There is substantial genetic and physiological data that show that Fpn is the iron exporter responsible for the entry of iron into plasma (Ganz and Nemeth, 2006). Expression of Fpn results in the loss of cellular iron including iron stored in ferritin (Nemeth *et al*, 2004). We show here that Fpn-mediated ferritin degradation occurs in the cytosol and requires the activity of the proteasome. Release of iron from ferritin occurs before ferritin degradation and does not require ubiquitination. Vertebrate ferritin chains expressed in bacteria can assemble a ferritin nanocage in the absence of iron (Santambrogio *et al*, 1993). Entry of iron into apoferritin can occur *in vitro* and depends primarily on the ferroxidase activity of the H-chain (Lawson *et al*, 1989). Thus, both ferritin assembly and iron incorporation are intrinsic properties of ferritin. Depletion of cytosolic iron *in vivo*, either through the action of Fpn, which exports iron from cells, or by Ccc1p, which imports iron into the yeast vacuole, leads to the loss of ferritin iron. The simplest interpretation of these findings is that iron entry and exit from ferritin is the result of an equilibrium based on the concentration of cytosolic iron. The observation that iron can exit ferritin in yeast, an organism that has no endogenous ferritin, suggests that specific machinery for ferritin iron release is not required. Given the presence of a reductant that converts Fe(III) within ferritin to Fe(II), iron release from ferritin may

be an autonomous property of ferritin, supporting the view that iron exits through pores in the ferritin nanocage (Jin *et al*, 2001).

Our data suggest that iron release from ferritin 'marks' the ferritin shell, designating it as a substrate for ubiquitination. We did not observe polyubiquitination of ferritin prior to ferritin degradation. Monoubiquitinated proteins are not normally recognized by the proteasome. We have not proven that monoubiquitination is a signal for proteasomal degradation. It may be that monoubiquitination is a signal for disassembly of ferritin nanocages and that the same modifications of ferritin that lead to monoubiquitination can also signal proteasomal degradation. Several reports have indicated that oxidized proteins including oxidized ferritin can be degraded by the proteasome (Rudeck *et al*, 2000; Mehlhase *et al*, 2005), without being polyubiquitinated (Shringarpure *et al*, 2003). The machinery that mediates ferritin disassembly is not specific to mammalian cells but is found in yeast. There is a robust literature showing that monoubiquitination of many membrane proteins is a signal for their internalization into the multivesicular body (for a review, see Hicke and Dunn, 2003). Monoubiquitination of histones is associated with transcriptional regulation in higher eukaryotes (Shilatifard, 2006) and with UV DNA repair (Matsushita *et al*, 2005). In these instances, monoubiquitination is not a prerequisite for degradation but rather is involved in targeting complexes that then perform enzymatic activities. Our data show that monoubiquitination of ferritin leads to its disassembly, suggesting that monoubiquitination may function in the disassembly of other supermolecular structures. The H-chain of ferritin contains 12 lysine residues and it is not clear which of the residues is ubiquitinated, or whether all of the residues are potential ubiquitination sites.

These results show that under conditions in which cytosolic iron is reduced by export (either into yeast vacuoles or out of mammalian cells by Fpn), ferritin degradation occurs by the proteasome. The cytosol of yeast and mammalian cells would certainly contain high levels of reducing agents (glutathione, NADPH). Evidence suggesting that cytosolic iron is in the form of Fe^{2+} is that reduced iron is the substrate for both vacuolar storage (Li *et al*, 2001) and mitochondrial heme synthesis in yeast (Lange *et al*, 1999) and ferritin storage in mammalian cells (Lawson *et al*, 1989). There is substantial data, including results shown here that indicate that ferritin can be degraded in lysosomes. Many of the studies that show ferritin degraded by lysosomes utilize conditions that may affect autophagy, such as amino-acid starvation (Ollinger and Roberg, 1997) or bacterial infection (Larson *et al*, 2004). The other condition that leads to lysosomal ferritin degradation is addition of iron chelators such as DFO, a bacterial siderophore (Bridges, 1987; Kidane *et al*, 2006). The ability of DFO to cross mammalian membranes is a subject of debate, and DFO may enter cells by endocytosis, localizing in lysosomes (Lloyd *et al*, 1991). DFO is used as a therapeutic agent to manage iron overload disease in humans. DFO cannot be absorbed through the intestine and must be injected. The concentration that is used to iron-deprive cultured cells (100 μM) is 10–20 times the maximal therapeutic plasma concentration of DFO (4–7 μM) (Porter, 2001), suggesting that DFO may be exhibiting a pharmacological effect on cultured cells, perhaps by inducing the lysosomal accumulation of iron. As shown here, there are circumstances in which DFO induced ferritin loss may also occur by proteasomal degradation. The observation that there is plasticity in the route in which ferritin can be degraded, cytosol versus lysosome, may explain pharmacological studies in which inhibitors of both lysosomal and proteasomal activities can affect ferritin degradation (Kwok and Richardson, 2004).

Expression of Fpn can deplete cells of ferritin iron, while inhibition of Fpn activity results in increased ferritin levels, suggesting that iron accumulation in ferritin may be a default pathway occurring when cytosolic iron levels are high. An equilibrium model of iron entry and exit into ferritin provides an explanation for regulation of intestinal iron uptake. Iron transported into intestinal absorptive cells can either accumulate in ferritin or be transported by Fpn across the basolateral membrane into plasma. The accumulation of intestinal ferritin has been referred to as the 'mucosal block', as this iron is lost from the body when the short-lived absorptive cells are lost from the intestine (Granick, 1951; Crosby, 1966). Ferritin iron accumulation is inversely correlated with Fpn, as expression of Fpn can deplete ferritin iron by lowering cytosolic iron and by exporting iron from cells. Fpn level may be one of the major determinants in regulating whether iron is stored in ferritin or made bioavailable. Accumulation of ferritin iron in mucosal cells (or in macrophages recycling red blood cell iron) may only occur in the absence of iron export and therefore may be a default pathway. Cell surface Fpn is regulated by hepcidin as binding of this peptide to Fpn induces degradation of the Fpn–hepcidin complex (Nemeth *et al*, 2004). Hepcidin produced in response to hepatic iron stores or inflammation can ultimately determine if cells export iron into plasma or store iron in ferritin.

Materials and methods

Cells and media

HEK293T-Fpn cells, a stable cell line in which Fpn-GFP is regulated by the ecdysone promoter, were grown as described (Nemeth *et al*, 2004). Fpn-GFP expression was induced by addition of 10 μM Ponasterone A (AG Scientific Inc., San Diego, CA). FM3A and ts85 were grown in RPMI 1640 (Gibco/Invitrogen, Grand Island, NY) with 5% fetal bovine serum as described (Ikehata *et al*, 1997). The *Saccharomyces cerevisiae* strains used in this study were derived from W303 background and have been used in previous studies (Li *et al*, 2001). Wild-type DY150, Δ*ccc1*, Δ*pep4* and *erg6-2* strains were transformed with pGAL, pGAL-L-ferritin, pGAL-H-ferritin and pGAL-H + L-ferritin vectors. The *erg6-2* strain was used as it permits the entry of the proteasome inhibitor MG132 (Lee and Goldberg, 1996). Cells were grown in yeast nitrogen base synthetic medium (CM) with supplements as needed.

Western blot analysis and immunoprecipitation

Cellular proteins were extracted with 150 mM NaCl, 10 mM EDTA, 10 mM Tris (pH7.4), 1% Triton X-100, a protease inhibitor cocktail (Roche, Palo Alto, CA) and 50 mM *N*-ethylmaleimide (Sigma, St Louis, MO). Total protein concentrations were determined using BCA reagent (Pierce, Rockford, IL). Protein samples were separated on 4–20% gels (Tris–glycine) (BioRad, Hercules, CA) and transferred on Hybond-ECL (Amersham Biosciences, Piscataway, NJ). Ferritin was detected using rabbit anti-ferritin antibody (1:1000, Sigma, St Louis, MO), with peroxidase conjugated goat anti-rabbit IgG as the secondary antibody (1:10 000, Jackson ImmunoResearch, West Grove, PA). Ubiquitination was detected using mouse anti-ubiquitin (1:1000, Covance, Berkeley, CA) with peroxidase conjugated goat anti-mouse IgG as the secondary antibody (1:10 000, Jackson ImmunoResearch, West Grove, PA). Chemiluminescent method was used for detection (Western Lightning, Perkin Elmer, Boston, MA). Ferritin was immunoprecipitated

using rabbit anti-ferritin antibody (1:250, Sigma, St Louis, MO) and Protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Samples were eluted from beads using 2 × SDS–PAGE sample buffer with β-mercaptoethanol. Prussian Blue staining of ferritin was performed as described (Kim *et al*, 2003).

Ferritin measurement

HEK293T-Fpn cells were incubated with FAC (10 μM Fe) and induced with 10 μM Ponasterone A. Cellular protein was extracted as described above. Ferritin levels from mammalian extracts were determined by an ELISA (Laguna Scientific, Laguna, CA), according to the manufacturer's instructions. Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL). Ferritin levels from yeast extracts were determined by an ELISA as described (Erhardt *et al*, 2004) using rabbit anti-ferritin antibody 0.05 mg/well. Error bars represent the standard deviation from three different experiments in duplicate.

Tf(^{59}Fe)$_2$ preparation

Human apoTf (Sigma, St Louis, MO) was iron loaded using either ^{59}Fe (New England Nuclear, Boston, MA) or ferric chloride as described (van Renswoude *et al*, 1982). Cells were incubated with 1.0×10^{-7} M Tf(^{59}Fe)$_2$.

Size exclusion chromatography

Cellular protein was extracted as described above and loaded on a Superdex 200 FPLC column (Amersham Pharmacia, Piscataway, NJ) that was standardized using thyroglobulin (330 kDa), alcohol dehydrogenase (150 kDa), BSA (67 kDa), ovalbumin (40 kDa), and cytochrome *c* (12.4 kDa, Sigma). Fractions were collected and analyzed for ferritin by ELISA or Western blot.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde, permeabilized in PBS containing 1% bovine serum albumin and 0.1% saponin and incubated in mouse anti-EGF-Receptor (1:100) (NeoMarkers, Fremont, CA) for 60 min at room temperature, followed by Alexa 594 conjugated goat anti-mouse antibody (1:750) (Molecular Probes, Eugene, OR) for 60 min at room temperature. Cells were visualized using an epifluorescence microscope (Olympus Inc., Melville, NY) with a × 100 oil immersion objective. Images were acquired using Magnafire analysis software (Optronix, Goleta, CA).

Percoll gradients and β-N-acetylhexoseaminidase analysis

Cells were homogenized in homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.5 mM EGTA, pH 7.2, KOH) using a ball-bearing homogenizer. Homogenates were centrifuged at 800 *g* for 5 min at 4°C to obtain a postnuclear supernatant, which was fractionated on 30% Percoll (Ward *et al*, 1990). Gradients were fractionated and β-*N*-acetylhexoseaminidase activity was assayed as described (Lamb *et al*, 1983) and ferritin measured by ELISA.

Measurement of iron by atomic absorption spectroscopy

Log phase cells were collected and washed by centrifugation with 50 mM Tris–HCl, pH 6.5, 10 mM EDTA. Cell pellets were digested in 200 ml of 5:2 nitric:perchloric acid at 80°C for 1 h. After digestion, the samples were diluted to 1.0 ml with deionized water and then flamed in a PerkinElmer Life Sciences inductively coupled plasma Atomic Absorption Spectrometer. All samples were measured in duplicate and normalized by total cell number.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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